FORM PTO-1390 (Modified) (REV 11-2000) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR
DC-0155	09/856749

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US99/26610	10 November 1999	30 November 1998

TITLE OF INVENTION

Methods of Diagnosing, Prognosticating and Treating Matrix Metalloproteinase-1 Related Diseases via a Matrix Metalloproteinase-1 Single Nucleotide Polymorphism

APPLICANT(S) FOR DO/EO/US

BRINCKERHOFF, Constance E. et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information.

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371
3. ☐ This is an express request to begin national examination procedures (35 U S.C. 371(f)). The submission must include items (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☐ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US)
6. ☐ An English language translation of the International Application as filed (35 U.S.C 371(c)(2)).
   a. ☐ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☒ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3 31 is included.
15. ☐ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☒ A computer-readable form of the sequence listing in accordance with
20. ☐ A second copy of the published international application under 35 U
21. ☐ A second copy of the English language translation of the internationa
22. ☐ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

1) Courtesy copy of International Application;
2) Statement to support filing and submission in accordance with 37 CFR 1.821-1.825 and amended sequence list
3) Return Post Card

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I hereby certify that this paper is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, Box PCT, Washington, D.C. 20231.

By Deborah Ehret
Typed Name: Deborah Ehret

531 Rec'd PCT/PTO 24 MAY 2001

U.S. APPLICATION NO (IF KNOWN, SEE 37 CFR)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/856749	PCT/US99/26610	DC-0155

24. The following fees are submitted..		CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :			
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO	**$1000.00**		
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO	**$860.00**		
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO	**$710.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4)	**$690.00**		
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4)	**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =		**$860.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).		**$0.00**	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	5 - 20 =	0	x $18.00	**$0.00**	
Independent claims	5 - 3 =	2	x $80 00	**$160.00**	
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$1,020.00**	
☒ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.				**$510.00**	
SUBTOTAL =				**$510.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$510.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3 28, 3.31) **(check if applicable).** ☒				**$40.00**	
TOTAL FEES ENCLOSED =				**$550.00**	
Applicant, Dartmouth College, is entitled to small entity status. It is a University or other Institute of higher education.				**Amount to be: refunded**	$
				charged	$

a. ☐ A check in the amount of _____ to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account No _____ in the amount of _____ to cover the above fees A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-1619** A duplicate copy of this sheet is enclosed.

d. ☒ Fees are to be charged to a credit card **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Jane Massey Licata, Reg. No. 32,257
Kathleen A. Tyrrell, Reg. No. 38,350
Licata & Tyrrell P.C.
66 E. Main Street
Marlton, New Jersey 08053

Telephone: (856) 810-1515
Facsimile : (856) 810-1454

Kathleen A. Tyrrell
SIGNATURE

Kathleen A. Tyrrell
NAME

38,350
REGISTRATION NUMBER

May 24, 2001
DATE

Methods of Diagnosing, Prognosticating and Treating Matrix Metalloproteinase-1 Related Diseases via a Matrix Metalloproteinase-1 Single Nucleotide Polymorphism

INTRODUCTION

This invention was made in the course of research sponsored by the National Institutes of Health. The U.S. Government may have certain rights in this invention.

BACKGROUND OF THE INVENTION

Matrix metalloproteinases (MMPs) are a family of at least 15 enzymes that degrade the extracellular matrix (ECM) (Borden, P. and Heller, R. Crit. Rev. Eukaryotic Gene Expr. 7: 159-178, 1997). These enzymes have essential roles in modeling and remodeling the ECM in normal physiology and disease pathology. Several of these enzymes have the unique ability to degrade the interstitial collagenase (types I, II, and III), the body's most abundant proteins. MMP-1 is the most ubiquitously expressed interstitial collagenase, thereby assigning it a prominent role in collagen degradation. Overexpression of MMP-1 is associated with several pathological conditions, including the irreversible degradation of cartilage, tendon, and bone in arthritis (Vincenti et al. Crit. Rev. Eukaryotic Gene Expr. 6:391-411, 1996) and the degradation of collagenase I and III in tumor invasion and metastasis (Chambers, A.F. and Matrisian, L.M. J. Nat'l Cancer Inst. 89:1260-1270, 1997; Murray et al. Nat.

Med. 2:461-462, 1996). Patients with tumors that express MMP-1 have an overall poorer prognosis than patients with tumors that do not express this protein (Murray et al. Nat. Med. 2:461-462, 1996; Murray et al. J. Pathol. 185:256-261, 1998). This overexpression of MMP-1 has been suggested to be due to the juxtaposition of transcription factor binding sites within the promoter of this gene and to the cooperativity among the factors that bind these sites (Buttice et al. Oncogene 13:2297-2306, 1996; Basuyaux et al. J. Biol. Chem. 272:26188-26195, 1997; Gutman, A. and Waslyk, B. EMBO J. 9:2241-2246, 1990; Benbow, U. and Brinckerhoff, C.E. Matrix Biol. 15:519-526, 1997).

Most normal cells express modest, but detectable, levels of MMP-1 constitutively, and this expression increases substantially in the presence of cytokines or growth factors (Vincenti et al. Crit. Rev. Eukaryotic Gene Expr. 6:391-411, 1996; Rutter et al. J. Cell Biochem. 66:322-336, 1997; Aho et al. Eur. J. Biochem. 247:503-510, 1997; Delany, A.M. and Brinckerhoff, C.E. J. Cell Biochem. 50:400-410, 1992). However, A2058 melanoma cells constitutively express high levels of MMP-1 (Templeton et al. Cancer Res. 50:5431-5437, 1990), making them a useful model for studies on the transcriptional regulating of this gene and for comparative studies with normal cells.

A 4 kb region of the MMP-1 promoter DNA from a leukocyte genomic library was isolated and sequenced (Rutter et al. J. Cell Biochem. 66:322-336, 1997). DNA sequence analysis revealed that this clone contained only 1 G at position -1607 bp, resulting in the sequence 5'-AAGAT-3' (SEQ ID NO: 1) (Rutter et al. J. Cell Biochem. 66:322-336, 1997). This sequence differs from that reported by others (Aho et al. Eur. J. Biochem. 247:503-510, 1997; Imai et al. Mol. Cell Biol. 14:7182-7194, 1994), wherein 2 Gs at that location which create the sequence 5'-AAGGAT-3' (SEQ ID NO: 2) are described. The presence of 2 Gs at this site creates the

sequence 5'-GGA-3', which is a consensus sequence (Graves, J.B. Science 279:1000-1001, 1998) for a functional PEA3/EBS. This site has now been established to constitute a single nucleotide polymorphism (SNP). The full length DNA sequence of MMP-1 with only 1 G at position -1607 is depicted in SEQ ID NO: 3.

Based upon the effect of this SNP on the transcriptional activity, protein/DNA binding activity, and frequency of this SNP in normal fibroblasts and in melanoma tumor cells it has now been determined that detection of this SNP is useful in diagnosing and prognosticating cancer and other MMP-1 related diseases. It is also now believed that agents identified as inhibitors of binding of transcription factors to the Ets transcription factor binding site created by or resulting from this SNP will be useful in treating MMP-1 related diseases.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method of diagnosing MMP-1 related diseases in a patient which comprises detecting in a patient MMP-1 containing an Ets transcription factor binding site single nucleotide polymorphism (MMP-1 EBS-SNP).

Another object of the present invention is to provide a method of prognosticating MMP-1 related diseases in a patient which comprises detecting in a patient suffering from an MMP-1 related disease an MMP-1 EBS-SNP.

Another object of the present invention is to provide a kit for diagnosis and prognosis of an MMP-1 related disease in a patient via detection of MMP-1 EBS-SNP in a patient sample.

Another object of the present invention is to provide a method of identifying agents with potential therapeutic value in treating MMP-1 related diseases which comprises screening agents for the ability to inhibit the binding of a transcription factor to an MMP-1 EBS-SNP.

Yet another object of the present invention is to provide a method of treating MMP-1 related diseases in a patient which comprises administering to the patient an agent which inhibits binding of a transcription factor to an MMP-1 EBS-SNP.

DETAILED DESCRIPTION OF THE INVENTION

The abundance of single nucleotide polymorphisms (SNPs) in the genome make these genomic variations powerful tools for identifying disease genes, particularly in loss of heterozygosity studies in tumors. A large effort is presently underway for identifying SNPs. Most research is focused on the coding regions of genes (Wang et al. Science 280:1077-1082, 1998).

However, an SNP located in the promoter of the interstitial collagen degrading enzyme, MMP-1, has now been identified. Because this variation is not located in a coding region, it does not alter the structure of the enzyme. However, the location of this SNP in the promoter region of MMP-1 has now been demonstrated to have profound effects on the production/regulation of the enzyme. Further, an increase in the frequency of this SNP in tumor cell lines has now been demonstrated which is indicative of this SNP causing an increase in invasive behavior due to high levels of MMP-1 expression. MMP-1 is implicated in tumor cell invasion and metastasis due to its ability to cleave the interstitial collagenase types I and III at neutral pH. Therefore, a structural variation with the potential to influence the level of expression is important to understanding how this enzyme modulates ECM metabolism and tumor cell invasion and metastasis. Further, detection of this SNP in a patient is useful in diagnosing and prognosticating MMP-1 related diseases.

The effects of this SNP, referred to herein as MMP-1 EBS-SNP, on the transcriptional activity and protein/DNA

binding activity, along with the frequency of this SNP in normal fibroblasts and in melanoma tumor cells were examined in detail.

Constitutive mRNA expression of endogenous MMP-1 gene in normal foreskin fibroblasts (HFS) and in the A2058 melanoma cells over a 24 hour period was determined. HFS expressed low levels of MMP-1 mRNA, while A2058 cells expressed higher levels. To determine whether the A2058 cells contained a difference in the endogenous MMP-1 promoter, polymerase chain reaction (PCR) was used to amplify the promoter from these cells. This sequence was then compared with the previously isolated leukocyte clone described by Rutter et al. J. Cell Biochem. 66:322-336, 1997 and set forth in SEQ ID NO: 3 and with promoters from other sources (Aho et al. Eur. J. Biochem. 247:503-510, 1997; Imai et al. Mol. Cell Biol. 14:7182-7194, 1994). Several substitutions were observed, but were considered insignificant because they did not create or delete any known binding sites for transcription factors within the promoter. However, one major difference was detected: the A2058 promoter DNA contained an additional G at position -1607 bp, which was flanked by a guanidine (5') and an adenine (3'), thus creating an Ets transcription factor binding site (EBS; Graves, B. Science 279:1000-1001, 1998).

To specifically test the role of the 1 G/2 G variation in regulating transcription, two luciferase reporter constructs driven by a large (4.3 kb) fragment of the MMP-1 promoter were generated with the only difference being 1 G or 2 Gs at -1607 bp. This SNP is adjacent to an AP-1 site -1602 bp, which may also influence transcription. These clones were transitely transfected in HFS and the effect on basal transcription was measured. A significant increase (ranging from 2-10-fold) in transcription with the 2 G promoter construct compared with the 1 G promoter construct was consistently observed in at least four separate donors of HFS. Hirt's analysis of transfected DNA demonstrated that these

differences were not attributable to transfection efficiency. When these two constructs were transfected into the A2058 melanoma cells, a 29-fold increase in transcription of the 2 G construct over the 1 G construct was observed. Other experiments showed similar increases ranging from 1- to 37-fold. These constructs were then tested in other tumor cell lines (MDA231 breast cancer cells, and two primary melanoma lines) to assess their transcriptional response. In these cells, the 2 G promoter construct augmented transcription at least 4-fold over the 1 G construct. Thus, the increase in transcription is dependent on the presence of 2 Gs at -1607 bp, and it is seen in both normal and malignant cells.

The ability of an oligo probe containing either 1 G or 2 Gs at -1607 bp to bind to nuclear extracts from the A2058 cells was also assessed. A striking difference in binding intensity was seen, with the 2 G oligo binding more nuclear proteins compared with the 1 G oligo. Thus, the presence of the 2 Gs clearly augments this binding. Cross competition experiments were used to determine the specificity of binding to each labeled oligo. Binding to the 1 G oligo was readily competed, suggesting that these DNA/protein interactions are weak. Competition studies with the 2 G oligo revealed that the "self" oligo only mildly competed the binding of the shared bands, although it did compete two bands, demonstrating that these proteins bind preferentially and/or specifically to the 2 G oligo . Furthermore, the 1 G oligo did not compete well, but was effective in competing the shared bands. Finally, the proximal EBS-AP-1 competitor showed a pattern similar to that seen with the 1 G oligo. Taken together, these observations indicate that the 2 G "self" oligo competes for the proteins able to bind specifically to the 2 gene sequence, and that the other bands represent proteins or protein complexes that are not 2 G-dependent (e.g. the AP-1 proteins). Importantly, these data also indicate that the

presence of the EBS in this region of MMP-1 creates an environment where DNA/protein interactions strongly occur.

Because the SNP at -1607 bp is located adjacent to an AP-1 site at -1602 bp, binding to the 1 G and 2 G oligos by recombinant ETS-1 and c-JUN, proteins that are likely candidates for binding to these sites, was investigated. The recombinant proteins, by themselves, were not able to bind to either oligo. The combination of both proteins, however, bound to the 2 G oligo, but only faintly to the 1 G oligo, suggesting that additional proteins were required for optimal DNA binding. Indeed, incubation with nuclear extract from A2058 cells resulted in distinctive binding patterns for each probe. Complexes I, II, VI, and VII were present in both panels, whereas complexes III, IV, and V were specific for the 2 G oligo. When ETS-1 and c-JUN were added together with nuclear extract, binding to the 2 G oligo was more pronounced compared with the 1 G oligo.

In the presence of recombinant c-JUN, complex I became more apparent with both oligos, indicating that complex I may represent AP-1 proteins binding to the DNA. Complex VII also became more intense when c-JUN was added indicating that it, too, contains AP-1 related proteins. When both recombinant proteins were added, the binding pattern in the 1 G panel did not differ from the reactions with c-JUN alone, supporting the importance of 2 Gs in creating the EBS. When nuclear extracts and recombinant ETS-1 were added to the 2 G oligo, a new complex was formed (complex IV). Furthermore, when both proteins and nuclear extract were added to the 2 G probe, several complexes (I, III, IV, V, and VI) were diminished and seemed to combine into a much stronger complex II, again demonstrating the influence of the 2 Gs in creating EBS.

Antibodies to several members of the Ets family of transcription factors (ETS-1/2, Erg-1/2, Elk-1 and ERM) were used in "super/shift" reactions to identify the proteins binding to this site. However, binding of these antibodies

was not detected due either to limitations of the antibodies, because the complexes did not allow for the antibody to have access to the epitope, and/or because these proteins did not bind. These data suggest that the oligos containing 2 Gs represents a bona fide EBS that binds an Ets family member(s) in a complex with AP-1 protein members. While the data show that recombinant ETS-1 binds, it is believed that other Ets family members can also bind to this site.

Confirmation of this 1 G/2 G difference in the leukocyte clone sequence and the A2058 melanoma sequence, to be a SNP and not a mutation, was performed. A radiolabeled PCR assay using primers that flank the variation to amplify a product of either 148 bp (1 G), 149 bp (2 G), or both (heterozygous) in genomic DNA was developed. One hundred control DNAs derived from the CEPH pedigrees (http://www.cephb.fr/cephdb/) were then assayed to determine the frequency of this variation within a population. Only the parents in the pedigrees were used to avoid biasing the results through inheritance. In addition to the CEPH control DNAs, the frequency of this SNP in several tumor cell lines, including the A2058 melanoma cells, was assessed. The occurrence of 2 G homozygotes in the CEPH controls was determined to be approximately 30%. In the tumor cells lines, it is 62.5% ($P<0.0001$).

Accordingly, based upon these experiments, it is believed that the 1 G/2 G SNP influences the transcriptional responsiveness of the human MMP-1 promoter in cancer, where excessive production of MMP-1 is a major contributor to the stromal degradation involved in tumor invasion. As with other genes, expression of MMP-1 is meditated by multiprotein complexes that bind to DNA in a sequence-specific manner, and these complexes often cooperate to achieve maximal activation. DNA elements containing a single EBS are often not sufficient for Ets induction, and require a nearby AP-1 site to which Fos and JUN proteins bind. Furthermore, the transcriptional environment within the cells may influence the level of gene

expression, as evidenced by differences in the level of transcription of the 2 G allele in HFS versus melanoma cells. These differences may be due to various extracellular stimuli, such as growth factors and cytokines, as well as cell-type-specific nuclear factors within the cell. Thus, the combination of cis-acting sequences in the MMP-1 promoter and specific trans-acting factors can dramatically increase transcription. This increase is believed to provide a molecular mechanism for enhanced ECM degradation not only in cancer, but in other MMP-1 related diseases such as arthritis, cardiovascular disease and periodontitis.

For example, rheumatoid arthritis, a chronic disease that afflicts more than two million individuals in the United Stated and 1% of the population world-wide is characterized by severe and irreversible degradation of cartilage, tendon and bone mediated by overexpression of MMPs. Inflammatory cytokines such as interleukin-1 and tumor necrosis factor-α activate synovial fibroblasts that line the joint and adjacent chondrocytes to produce these MMPs. Destruction of interstitial collagen occurs mainly through two MMP family members, MMP-1 and MMP-13. MMP-1 and MMP-13 are expressed by synovial fibroblasts and articular chondrocytes. It is believed that, in similar fashion to patients suffering from cancer, overexpression of MMP-1 in patients suffering from rheumatoid arthritis may result at least in part from this SNP.

Accordingly, detection of the MMP-1 EBS-SNP in a patient provides a useful means for diagnosing MMP-1 related diseases. Detection of MMP-1 EBS-SNP in a patient is indicative of the patient suffering from a disease relating to overexpression of the MMP-1 enzyme. Detection of this SNP can be performed in accordance with well known techniques including, but not limited to, PCR as described herein.

Detection of MMP-1 EBS-SNP also provides a useful method for prognosticating MMP-1 related diseases in a patient. For

example, it is well known that MMPs are key players in tumor invasion and metastasis. Both processes require degradation of the extracellular matrix, which is accomplished by proteolytic enzymes that are secreted by either tumor cells, themselves, and/or neighboring stromal cells. In fact, at least for melanoma cells which produce MMP-1, the prognosis of the disease is correlated with tumor thickness and depth of invasion through dermal collagens (I and II), thereby indicating that invasiveness through these collagens serves as a valid prognostic marker. Accordingly, detection of MMP-1 EBS-SNP, which is indicative of enhanced ability to degrade collagen types I, II and III, in tumor cells of a patient serves as a useful prognostic marker in assessing the invasiveness of a particular tumor. This prognostic marker is thus useful in determining various treatment regimes expected to be most successful in individual patients.

Means for detecting MMP-1 EBS-SNP in a patient sample for diagnosing and/or prognosticating MMP-1 related diseases can be incorporated into a kit for easy use by a laboratory technician. In one embodiment, the kit can comprise PCR primers such as those described in Example 5 herein which flank the MMP-1 EBS SNP. In this embodiment, the kit may also comprise dGTP, dATP, dTTP, and dCTP; Taq DNA polymerase; and α(32)P-dCTP. However, other means for detecting the MMP-1 EBS-SNP which can be incorporated into a kit will be obvious to those of skill in the art upon this disclsoure. Kits of the present invention may also comprise positive and negative control samples.

Further, inhibiting MMP-1 synthesis by targeting either the MMP-1 EBS-SNP or proteins that bind to this SNP represents a useful therapeutic approach to inhibit pathologic expression of MMP-1, but not its normal physiological role. Potential therapeutic agents for treatment of MMP-1 related diseases including, but not limited to, cancer, rheumatoid arthritis, cardiovascular disease and periodontitis, can be identified

by determining their ability to either bind to MMP-1 EBS-SNP, to bind to proteins which bind to MMP-1 EBS-SNP or to inhibit binding of MMP-1 EBS-SNP with other proteins. Agents identified by this method can then be administered to patients suffering from an MMP-1 related disease to alleviate the symptoms resulting from overexpression of MMP-1.

The following nonlimiting examples are provided to further illustrate the present invention.

EXAMPLES

Example 1: Cells and Plasmids

Primary human foreskin fibroblasts (HFF) were prepared in accordance with procedures described by Rutter et al. J. Cell Biochem. 66:322-336, 1997, and used during passages 4-8. HFFs and A2058 cells were cultured in DMEM (Life technologies, Inc.) with 10% fetal bovine serum (Sigma Chemical Co.), penicillin (100 units/ml), and streptomycin (100 µg/ml).

A 4.3-kb MMP-1 promoter DNA fragment containing only 1G at -1607 bp was described by Rutter et al. J. Cell Biochem. 66:322-336, 1997. Primers were made to amplify the endogenous promoter from the A2058 cells (-4008 bp to -3988 bp sense primer: 5'-GTGGAAGCTTACACCTATAATCCCAACACTC-3' (SEQ ID NO: 4) and -511 bp to -543 bp antisense primer: 5'-CTGCCTGGTACCCTATTGCGATAGCACCATGGC-3' (SEQ ID NO: 5). Two A2058 PCR amplified clones were subcloned into the pBL5CAT (Promega) vector and sequenced to ensure the absence of PCR artifacts. Reporter clones were then constructed in which the only difference between the two pGL3-MMP-1 vectors was the SNP at position -1607 bp. First, the MMP-1 promoter insert from the pXP2 vector was subcloned into the pGL3 Basic vector (Promega). Unique sequences flanking the G variation were restricted by AatII (5') and EcoRV (3'), thereby isolating a 450/451-bp fragment from the leukocyte clone in pGL3 Basic (1 G) and A2058 melanoma DNA in pBL5CAT (2 Gs), respectively, and excluding any other sequence variations found in the A2058 promoter. Finally, the 451-bp insert containing the 2 Gs was

"swapped" and ligated into the pGL3-MMP-1 construct containing AatII/EcoRV ends, thereby generating the two SNP constructs.

Example 2: Northern Analysis

Confluent cultures in 150-mm diameter tissue culture dishes were washed and placed in 10 ml of serum-free DMEM plus 0.2% lactalbumin hydrolysate. Immediately (time 0) and at 24 hours, total RNA was isolated using the TRIzol reagent (Life Technologies, Inc.) and 20 µg were subjected to Northern analysis and hybridized with cDNA-specific probes for MMP-1 or glyceraldehyde-3-phosphate dehydrogenase. Probes were random prime labeled with α(32)P-dCTP (12.5 µCi/reaction of 3,000 Ci/mmol) and hybridized for 20 hours at 56°C.

Example 3: Transfection and Luciferase Assay

Transient transfections were performed in triplicate in accordance with procedures described by Rutter et al. J. Cell Biochem. 66:322-336, 1997 with the LipofectAMINE PLUS reagent (Life Technologies, Inc.) using 2 µg of the chimeric MMP-1 promoter/reporter plasmids, 5 µl of the PLUS reagent, and 5 µl of LipofectAMINE. Luciferase activity is reported as RLUs. Hirt's analyses were performed and normalized to RLUs to control for any variations in transfection efficiency. Statistics were performed using the InStat Program (GraphPad Software) using the Welch's alternate t test, a modification of the unpaired t test.

Example 4: Nuclear Extract Preparation and EMSAs

Extracts of nuclear proteins were prepared, and EMSAs were performed as described by Schroen, D.L. and Brinckerhoff, C.E. J. Cell Physiol. 169:320-332, 1996, with 1 x 10^5 cpm of γ^{32}P-ATP end-labeled oligo incubated with nuclear extract (5 µg) and/or recombinants ETS-1 protein (2 µM) and c-JUN protein (1 µg; Promega). The samples were subjected to 5% PAGE at 150 V, dried, and autoradiographed. Oligos used for EMSAs were

1G sense, 5'-AAATAATTAGAAAGATATGACTTATCTCAAATCAA-3' (SEQ ID NO: 6); 2 G sense, 5'-AAATAATTAGAAAGGATATGACTTATCTCAAATCAA: (SEQ ID NO: 7) -88/-73 sense, 5'-TTCATTGTTAATCAAGAGGATGTTATAAAGCATGAGTCACACCCTCAGCTT-3' (SEQ ID NO: 8). The -88/-73 oligo spans the region -110 to -61 bp and includes the locations within the oligo that correspond to the proximal PEA3/AP-1 sites at the -88/-73, respectively.

Example 5: Radiolabeled PCR Assay

Primers that flank the SNP in MMP-1 were used for PCR amplification (sense primer, 5'-GTTATGCCACTTAGATGAGG-3' (SEQ ID NO: 9); antisense primer 5'-TTCCTCCCCTTATGGATTCC-3' (SEQ ID NO: 10)). A typical reaction consisted of ~20 ng of DNA template; 0.2 mM dGTP, dATP, and dTTP, 2.5 µM dCTP; 10 x buffer and Taq DNA polymerase (Sigma Chemical Co.); and α(32)P-dCTP (DuPont/NEN). Reactions were PCR amplified (MJ Research PTC100 THERMOCYCLER) in 25 cycles (4 minutes at 94°C; 45 seconds at 94°C, 45 seconds at 58°C, and 45 seconds at 72°C; followed by a brief extension (10 minutes) at 72°C). Following amplification, the reactions (2.5 µl) were mixed with 10 X loading buffer and denatured for 2 minutes at 80°C. Samples were loaded onto an 8% denaturing PAGE and electrophoresed for 3 hours. Gels were dried and autoradiographed for approximately 15 minutes. Control samples generated from the plasmid clones were loaded on each gel for accurate scoring of the alleles. Statistics were calculated using the InStat Program (GraphPad Software) and were based on the percentage of the total number tested.

What is claimed is:

1. A method of diagnosing a matrix metalloproteinase-1 related disease in a patient comprising detecting in the patient matrix metalloproteinase-1 containing the Ets transcription factor binding site single nucleotide polymorphism.

2. A method of prognosticating a matrix metalloproteinase-1 related disease in a patient suffering from a matrix metalloproteinase-1 related disease comprising detecting in the patient matrix metalloproteinase-1 containing the Ets transcription factor binding site single nucleotide polymorphism.

3. A kit for diagnosing and prognosticating MMP-1 related diseases in a patient sample comprising a means for detecting matrix metalloproteinase-1 containing the Ets transcription factor binding site single nucleotide polymorphism in a patient sample.

4. A method of identifying potential therapeutic agents for treatment of a matrix metalloproteinase-1 related disease comprising determining a potential therapeutic agent's ability to inhibit binding of a transcription factor to an Ets transcription factor binding site of single nucleotide polymorphism matrix metalloproteinase-1.

5. A method of treating patients suffering from a matrix metalloproteinase-1 related disease comprising administering to the patient an agent which inhibits binding of a transcription factor to an Ets transcription factor binding site of single nucleotide polymorphism matrix metalloproteinase-1.

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(71) Applicant *(for all designated States except US)*: TRUSTEES OF DARTMOUTH COLLEGE [US/US]; Technology Transfer Office, 11 Rope Ferry Road #6210, Hanover, NH 03755–1404 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BRINCKERHOFF, Constance, E. [US/US]; 7 Falcon Lane, Norwich, VT 05055 (US). RUTTER, Joni, L. [US/US]; Balley Road, Thetford, VT 05074 (US).

(74) Agents: LICATA, Jane, Massey et al.; Law Offices of Jane Massey Licata, 66 E. Main Street, Marlton, NJ 08053 (US).

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(54) Title: METHODS OF DIAGNOSING, PROGNOSTICATING AND TREATING MATRIX METALLOPROTEINASE–1 RELATED DISEASES VIA A MATRIX METALLOPROTEINASE–1 SINGLE NUCLEOTIDE POLYMORPHISM

(57) Abstract

Methods and kits for diagnosing and prognosticating matrix metalloproteinase–1 related disease by detecting a single nucleotide polymorphism in the promoter of the gene are provided. Also provided are methods of identifying agents which inhibit binding of transcriptions factors to the Ets transcription factor binding site created by or resulting from this single nucleotide polymorphism and methods of using these agents to treat matrix metalloproteinase–1 related diseases.

Docket No.
DC-0155

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Methods of Diagnosing, Prognosticating and Treating Matrix Metalloproteinase-1 Related Diseases via a Matrix Metalloproteinase-1 Single Nucleotide Polymorphism

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on **10 November 1999** as United States Application No. or PCT International Application Number **PCT/US99/26610**

and was amended on _____
(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐
(Number)	(Country)	(Day/Month/Year Filed)	☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

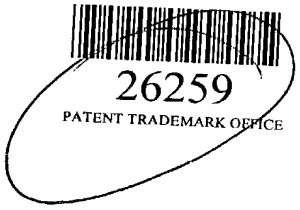
60/110,266	November 30, 1998
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*



Send Correspondence to:

Direct Telephone Calls to: *(name and telephone number)*
Jane Massey Licata or Kathleen A. Tyrrell - (856) 810-1515

Full name of sole or first inventor **Constance E. Brinckerhoff**
Sole or first inventor's signature Constance E. Brinckerhoff Date 5/14/01
Residence **Norwich, Vermont** VT
Citizenship **US**
Post Office Address **7 Falcon Lane**
Norwich, Vermont 05055

Full name of second inventor, if any **Joni L. Rutter**
Second inventor's signature J. L. Rutter Date 5.14.01
Residence ~~**Silverspring, Maryland**~~ Silver Spring, Maryland MD
Citizenship **US**
Post Office Address **3718 Lamberton Square Road, Apt. 1735**
~~**Silverspring, Maryland 20904**~~ Silver Spring, Maryland 20904

SEQUENCE LISTING

<110> Brinckerhoff, Constance
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Trustees of Dartmouth College

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